On the Reproduction of Kalpidorhynchus arenicolæ (Cnghm.).

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With Plate 29.

Introduction.

In 1907 Mr. Cunningham described and gave a life-history of this gregarine in the 'Archiv für Protistenkunde.' The parasite was first noticed by Mr. De Morgan while dissecting some specimens of Arenicola ecandata in this laboratory. Owing to pressure of other work Mr. Cunningham was unable to give a complete account of the reproduction, and he therefore suggested that I should, at some future time, try to find the first division nucleus with a view to ascertaining where the chromatin of its chromosomes came from. The latter half of this problem still remains unsolved; but after cutting many cysts into sections I did find the first spindle in a very early state, and I have been able to make one or two other observations which may prove to be not without interest.

Methods.

The cysts were fixed with various fluids, Hermann's, Flemming's, corrosive sublimate and acetic acid, with and without the addition of formaline, Bonin's picro-formol and Brasil's picro-formol. The best results were obtained by using the picro-formol mixtures. The sections were stained

by Heidenhain's method. Following the directions given by Brasil (1905), I left them for twenty-four hours in the mordant, and for thirty-six or more in the hæmatoxylin. As plasma stains after Heidenhain I used a mixture of Licht-Grün and picric acid in equal parts dissolved in absolute alcohol, orange G., and eosin.

Two other stains of which I made use were Delafield's hæmatoxylin and that of Kleinenberg.

The First Spindle.

The nuclear membrane disappears gradually bit by bit, and the nucleus becomes more or less diffuse, assuming an irregular outline in the parts no longer contained by the membrane. Close to this uneven edge there appears a little cluster of rod-like pieces of very darkly staining chromatin. These small rods are connected together by a fine thread, which stains more faintly than they do, so as to form a small, loosely folded skein. Part of this skein rests on the achromatic fibres of the spindle. These fibres are doubtless intranuclear in origin. The spindle has centrioles which stain deeply with Heidenhain's hæmatoxylin; and there are the usual terminal radiations in the cytoplasm (fig. 1).

In my next stage, which follows very closely upon the above, the nucleus is almost completely dissolved, all that remains of it being two or three karyosomes still unabsorbed by the cytoplasm. The spindle is now in anaphase (fig. 2). It shows a good number of deflected radiations, in this much resembling one figured by Brasil (1905), but here one can see a well-marked centrosome. I could not demonstrate a centriole. The number of the chromosomes is four. In metaphase (fig. 3) it is impossible to count the chromosomes, and there appear to be more than four, but in the anaphase they can generally be counted, and as I have seen numbers of nuclei in this phase I am in no doubt as to the number.

This appearance of the first spindle agrees in all essentials with the events described by Cuénot (1900), Brasil and

others in the formation of the first spindle in the Gregarines; but most closely with the facts in Monocystis ascidiæ (R. Lank.), Siedlecki (1900), and those in G. ovata as described by Schnitzler (1905), for here there is no vesicle.

The divisions proceed very rapidly; still, I was fortunate enough to find a cyst with only two nuclei, both, however, dividing (fig. 3).

In a stage with a small number of nuclei it could be seen that in the late anaphase the spindles stretch out, and become, consequently, very much attenuated in the middle. Thus the two daughter-nuclei arising from a division seem, at any rate during the rearrangement of the chromatin, to be surrounded and supported by the wide ends of the spindle of the mother-nucleus (figs. 4 *a* and *b*). Siedlecki (1900) and Brasil record the same method of division in Monocystids, and Brasil says that it seems as though the fibres of the spindle helped largely in forming the membranes of the daughter-nuclei. I did not notice that division went on more rapidly at the periphery of the cyst than elsewhere.

In these early stages the centrosomes could be easily demonstrated, and sometimes a sphere could be seen lying completely within the nuclear membrane showing its intranuclear origin (fig. 5). It would seem, too, that the spindle in these divisions is formed within the nuclear membrane, which only disappears when the spindle is fully formed (figs. 5 *a* and 5 *b*). Most of the nuclei contain two or three, some four, spherules of chromatin. These are probably karyosomes, though on account of their small size it is impossible to demonstrate two layers in even the largest of them.

In a few of the cysts, all of about the same age, i.e. having approximately the same number of nuclei, a small sphere of chromatin could be seen being thrust out of each nuclear spindle during division. It is worthy of note that if one spindle in a cyst showed this every other spindle in that cyst did so too. This cannot be regarded as a case of Reduction, for the number of the chromosomes remained

unchanged. I look upon it merely as the casting out of superfluous chromatin, most probably one of the spherules mentioned above, which is quickly dissolved and then absorbed by the surrounding protoplasm (fig. 5, *sph.*).

In some of the cysts intermediate in age between that shown in fig. 5 and the pearl stage there are to be seen some large nuclei at least twice the size of the others. I have never seen these nuclei dividing, but some of them are in a state of degeneration. In the earlier stages all the nuclei divide in exactly the same way. There are not two methods of division as in Stylorhynchus (Léger, 1903). These nuclei in the earlier stages seem to be not in any way different from their neighbours; but after having divided a certain number of times they divide no more, then degenerate and die. In the process of degeneration they naturally swell up a little, but their largeness in size as compared with the others is mainly due to their not having undergone so many divisions.

It seems to me that mitotic divisions are continued right on until the "pearl stage," or, as Mr. Cunningham calls it, the "convolution stage" is reached.

Mr. Cunningham, when he wrote his account of Kalpidorhynchus, was inclined to think that he was dealing with a case of isogamy. Nevertheless, he found a slight difference between the contents of the gametocytes enclosed in one cyst. I, too, am convinced that there must be an inherent difference between the gametocytes, but in the cysts which I cut this difference was not appreciable till just before the pearl stage was reached. Then, while the dividing-wall between the two gametocytes was still intact, it could be seen that the protoplasm on one side of the wall stained more deeply than that on the other, and that its meshwork was slightly, but only very slightly, finer. It could also be seen that the nuclei on the darker side were rounder, smaller, and darker than the others, showing a more concentrated chromatin. In sections through cysts at the pearl stage a great accentuation of these differences can be noticed. On

the dark or female side the chains of pearls lie on the edges of convoluted bands, which have about half the depth of those on the male side, showing that in the male gametocyte there is much more protoplasm left over after the formation of the gametes than in the female. The nuclei also can be seen in many cases to be about twice as large on the pale as on the dark side (fig. 6). This convinced me that we have anisogamy here, the chief thing which led me to this conclusion being the difference in size between the nuclei of the gametes in the respective gametocytes. But it would not have been easy to prove this difference by means of sections only, for the cysts were cut in all manner of planes. I therefore broke numbers of cysts at random on different slides, and was fortunate enough to isolate in this way a few female gametes from the "pearl stage" and a number of conjugation stages from cysts containing conjugating gametes. Unfortunately I did not so isolate a male gamete, but in the conjugation stages one could see its shape perfectly well.

The female gamete is nearly spherical in shape with a spherical nucleus, which, as a rule, has darker and more concentrated chromatin than that of the male gamete. This nucleus has a volume equal to about one fourth of that of the whole gamete.

The male gamete, on the other hand, consists almost entirely of an oval, sometimes of a pyriform nucleus, which is surrounded by a very thin layer of protoplasm. This nucleus is generally about twice as large as that of the female gamete. The oval nuclei are the more common (fig. 7).

While the nucleus of the female gamete is surmounted by a wide, low cone, the cone on the male nucleus is high and narrow. In both cases the centrioles can often be seen to be double.

In the act of conjugation it seems as though the male nucleus with its cone forces itself through its own protoplasm, which it casts off like a sheath as it enters the female gamete (fig. 7, *b*, *c*, *d*, etc.). This is what happens most frequently,

but I found some conjugations in which there was apparently a fusion of the cytoplasm of the gametes, as well as of their nuclei.

In neither gamete nor zygote could I demonstrate a cell-wall by the use of Delafield's hæmatoxylin, but preparations stained with Licht-Grün and picric acid showed a delicate outline to the cells. This outline was more easily shown in sections than in whole cell preparations.

The zygote is at first pyriform with very little in the way of a stalk, but with one end a good deal thicker and rounder than the other. The cell-wall is slightly more pronounced than it was in the conjugation stage. It is at the narrow, pointed end of the zygote that its nucleus lies. This nucleus is also pyriform and has its wide end directed towards the wide end of the zygote. Its chromatin is loosely arranged in large thick rods and lumps, and is not surrounded by a membrane. The absence of a nuclear membrane here is probably not merely a result of the fusion of the nuclei, but also a means of aiding the expulsion of a vacuole from the nucleus (fig 8, *b*).

Brasil (1905) also notes the expulsion of a vacuole (sphére hyaline) from the nucleus of every zygote in a cyst; and, as well as the vacuole, he saw extruded a small globule of chromatin, which he conjectures may form part of that chromatin which is subsequently to be seen at both ends of the spores of Monocystis after the first nuclear division. I saw no such extrusion of a grain of chromatin here, but on the assumption that it is merely superfluous chromatin this is not to be wondered at, for a small globule of superfluous chromatin was ejected at an earlier stage (see above and fig. 8).

The absence of a nuclear membrane may possibly facilitate the movements of the nucleus, for it certainly does move. One can see the vacuole forming, and after its extrusion the nucleus has not only acquired a membrane, but now lies at the wide end of the zygote. Nuclei can be seen in intermediate positions during the formation of the vacuole. Its

extrusion and the formation of the membrane seem to take place simultaneously.

After this the nucleus becomes approximately spherical, and its chromatin appears to be more finely divided and more closely packed than it was. The zygote at this stage often has a stalk-like projection at its narrow end, and this stalk persists so that the spore has the shape of a pear with a thickened stalk. Sometimes this stalk-like projection or elongation does not appear till later, but it is invariably present in the stage with four nuclei. The nucleus now divides into two, then into four, and ultimately into the eight nuclei of the sporozoites. It is my belief that at any rate the earlier of these divisions are mitotic, but I have not been able to prove this satisfactorily, and Mr. Cunningham is not of my opinion. It is at the stage with one spherical nucleus at the wide end of the cell that the cell-wall becomes thickened to form the sporocyst, and the zygote thus becomes a spore. Mr. Cunningham has mentioned the transparency of this sporocyst. I was able to see it, in preparations stained and mounted in spirit, as a dark line which follows the outline of the cytoplasm very closely. Rarely, until the spores are fully ripe, i. e. until the cytoplasm is segregated round each of the sporozoite nuclei, does it leave the little stalk-like projection of the sporocyst (fig. 8).

In some of my preparations the ripe spores are burst. This may be due to reagents, the withdrawal of the cytoplasm from the stalk having left a spot vulnerable to pressure in the sporocyst. But it may be the natural course of events, for the sporocyst seems to fit the cytoplasm fairly tightly, and the withdrawal of some of the cytoplasm from the stalk into the body of the spore may have caused the sporocyst to split.

The sporozoites are vermiform, with pointed ends. The long nucleus occupies at least half the volume of each individual. The chromatin is finely divided and evenly distributed throughout the whole nucleus (fig. 8).

It seems to me that it is only by following this chromatin

in the nuclei of the stages between sporozoite and trophozoite, and in watching the evolution of the karyosomes, that we can arrive at any conclusion as to the origin of the chromatin of the chromosomes in the first spindle. With this end in view I examined the alimentary tract of several infected specimens of Arenicola ecaudata, and found cysts in the œsophagus and in the intestine, which shows that swallowing is a possible method of infection. I have also cut sections through the gut walls of several specimens in the hopes of finding sporozoites in transit, but always without success. It seems most likely that the sporozoites make their way very speedily through the gut walls and then carry on their further development in the cœlomic fluid. I did find in the cœlom specimens of a very young trophozoite without an epimerite (fig. 9); but this, unfortunately for my purposes, had already several large and small karyosomes in its nucleus.

Two other points on which I have been able to supplement Mr. Cunningham's observations are multiple association and the structure and reproduction of the karyosomes.

Multiple Associations.—In preparing the cysts for embedding I noticed many cases of multiple association—sometimes there were as many as five individuals together, sometimes four, and very frequently three. Mr. Cunningham has figured four trophozoites coming together (1907). On cutting the sections I found a cyst containing five gametocytes, each of which had many nuclei; and I found several cysts with three gametocytes in like condition. But since I have never found a cyst containing more than two gametocytes in the "pearl stage" or further advanced, I am forced on to Dr. Woodcock's conclusion (1906) that these multiple associations come to nothing.

Structure of the Karyosome.

Unfortunately I have not succeeded in tracing the origin of the first karyosome. In the youngest trophozoite seen by me (fig. 9) there are already several karyosomes in the nucleus.

The larger karyosomes all consist of two layers—an outer dense layer which stains deeply and strongly with Heidenhain's hæmatoxylin and other chromatin stains, and is, in fact, basophile, and an inner part which has not so strong an attraction for basic stains, stains palely with Heidenhain, and strongly with acid stains such as orange G., Licht-Grün and picric acid, etc. The outer layer is, of course, chromatin, and the inner is nucleolar substance or plastin. The smaller karyosomes contain no plastin. They consist wholly of basophile chromatin. This, it seems to me, is only an expression of the fact that, as the karyosome increases in age and size, it becomes by degrees converted from a basophile into an acidophile substance—i.e. from chromatin into plastin. The larger karyosomes are often divided up into a number of small chambers, each chamber being surrounded by a wall or walls of chromatin. In fig. 10 there is a karyosome in which this process is beginning. This drawing shows the nucleus of a gametocyte, but the nuclei of trophozoites often contain karyosomes in the same condition. There can be little doubt that the chromatin of the partitions and of the little knob-like thickenings is derived from the dense outer layer. This turning in, so to speak, of the chromatin may take place in order to increase its area of action, for the result is an increase in the quantity of the plastin, and ultimately this increase is at the expense of the chromatin. I have seen karyosomes in which there were larger chambers with thinner walls, and others from which the outer rim of chromatin had gone completely. It seems as though finally the whole karyosome becomes converted into plastin.

The karyosome divided up into chambers resembles on a small scale the karyosome of Aggregata as described by Moroff (1908).

Reproduction of the Karyosomes.

Increase in the number of karyosomes takes place by a kind of internal budding from the chromatin layer. I have

not seen a single case of scissiparity. The buds (fig. 10) consist entirely of chromatin, and it is not until some time after their escape from the parent karyosome that the inner layer (plastin) makes its appearance in them.

On first noticing these internal buds I was puzzled as to how they made their escape, but soon came to the conclusion that an exit was made as occasion demanded and then closed up again. I was therefore much pleased to find that Schneider had described the same kind of internal budding in his account of Klossia (Aggregata) eberthi as long ago as 1883. See also Schellack (1907).

At present, affinity for different stains is our usual criterion for differentiating the contents of the cell, and we make a broad distinction between chromatin and cytoplasm by saying that one is basophile and the other acidophile.

In working at this gregarine my first staining operation was generally the use of Heidenhain's hæmatoxylin, and I could not help noticing that in staining strongly with an acid stain, after using Heidenhain, the inner part of the karyosome, the linin meshwork and the centrosomes all took up the acid stain (eosin, orange G., or picric and Licht-Grün), appearing to be stained by that and by nothing else. The centrioles and outer layer of the karyosomes and the chromosomes, however, kept black and were not affected by the acid stain at all. If I stained weakly with the acid stain the meshwork, inner part of the karyosome, and the centrosomes all retained the black stain of the Heidenhain, though the black on the inner part of the karyosome might with more accuracy be called grey. I could, in fact, vary the amount of greenness or blackness by varying the intensity of my acid stain, but one or other always predominated. Now the linin meshwork is known to consist partly of protoplasm and partly of chromatin. In the inner part of the karyosome chromatin is being converted into plastin, presumably for the nourishment of the nucleus and ultimately of the cytoplasm. Does it not seem that while this process of conversion in going on, there must be in the inner part of the karyosome a mixture of

acidophile plastin and basophile chromatin, and that in the centrosomes also there is a mixture of chromatin and an acidophile substance? On this supposition we can, at least, give our explanation of the results of staining; for it would seem that when (after using Heidenhain) we stain strongly with an acid stain, then in the resulting preparation the protoplasm masks the chromatin; on the other hand, when (after Heidenhain) we stain weakly with an acid stain or do not use one at all, the chromatin masks the protoplasm. The chromosomes, centrioles and outer part of the karyosomes, since they consist entirely of chromatin, when once stained with Heidenhain keep their black appearance unaltered by any subsequent treatment with acid stains.

In conclusion, I wish to express my thanks to Professor Minchin for his friendly advice as to literature, and also for his criticism of this paper.

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EXPLANATION OF PLATE 29.

Illustrating Miss M. Robinson's paper "On the Reproduction of Kalpidorhynchus arenicolæ (Cnghm.)."

Fig. 1.—The first nuclear spindle and the breaking up nucleus of the gametocyte. × 1200.

Fig. 2.—The first nuclear spindle. Anaphase. × 1050.

Fig. 3.—The second and third nuclei. Metaphase. × 1050.

Fig. 4.—(*a*) Spindle drawn out during late anaphase. (*b*) Two daughter-nuclei resulting from above. × 1200.

Fig. 5.—Part of a cyst in section, showing nuclei in different states of division. × 1200.

Fig. 5*a* and 5*b*.—Two nuclei from same cyst as fig. 5, showing formation of the spindle within the nuclear membrane. × 1200.

Fig. 6.—Portion of cyst in section at the pearl stage. × 500.

Fig. 7.—(*d*) Female gamete. × 1250. (*b*—*g*) Conjugation stages stained with Heidenhain, Licht-Grün and picric acid. × 1250. (*h*—*m*) Conjugation stages stained with Delafield's hæmatoxylin. × 1250.

Fig. 8.—(*a*) Zygote soon after conjugation. (*b*) Zygote with nucleus extruding a vacuole. (*c*) Spore—one nucleus stage. (*d*) Spore with two nuclei. (*e*) Spore with four nuclei. (*f*) Spore with eight nuclei. (*g*) Spore with eight nuclei. (*h*) Sporozoite. × 2000.

Fig. 9.—Young trophozoite without an epimerite. × 500.

Fig. 10.—Nucleus of a gametocyte showing multiplication of the karyosome, and division of a karyosome into chambers. × 2000.